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CC-Chemokine mutants against liver diseases

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CC-CHEMOKINE MUTANTS AGAINST LIVER DISEASES

FIELD OF THE INVENTION

The present invention relates to novel therapeutic applications of CC-chemokine mutants.

BACKGROUND OF THE INVENTION

Chemokines are secreted pro-inflammatory proteins of small dimensions (70-130 amino acids) mostly involved in the directional migration and activation of cells, especially the extravasation of leukocytes from the blood to tissue localizations needing the recruitment of these cells (Baggiolini M et al., 1997; Rossi D and Zlotnik A, 2000; Fernandez EJ and Lolis E, 2002). Usually chemokines are produced at the site of an injury, inflammation, or other tissue alteration in a paracrine or autocrine fashion, triggering cell-type specific migration and activation.

Depending on the number and the position of the conserved cysteines in the sequence, chemokines are classified into C-, CC-, CXC- and CX_3C-chemokines. Inside each of these families, chemokines can be further grouped according to the homology of the entire sequence, or of specific segments.

A series of heptahelical G-protein coupled membrane receptors, are the binding partners that allow chemokines to exert their biological activity on the target cells, which present specific combinations of receptors according to their state and/or type. An unified nomenclature for chemokine ligands and receptors, which were originally named by the scientists discovering them in a very heterogeneous manner, has been proposed to associate each of these molecule to a systemic name including a

progressive number: CCL1 CCL2, etc. for CC chemokines; CCR1 CCR2, etc. for CC chemokines receptors, and so on.

The physiological effects of chemokines result from a complex and integrated system of concurrent interactions. The receptors often have overlapping ligand specificity, so that a single receptor can bind different chemokines, as well a single chemokine can bind different receptors. In particular, N-terminal domain of chemokines is involved in receptor binding and N-terminal processing can either activate chemokines or render chemokines completely inactive.

Amongst all the chemokines characterized so far, CC-chemokines, such as CCL5 (also known as RANTES; Appay V and Rowland-Jones SL, 2001) have been intensively studied to identify therapeutically useful molecules. Variants of CC-chemokines, missing up to nine N-terminal amino acids, have been tested for their activity as inhibitors or antagonists of the naturally occurring forms. These molecules are inactive on monocytes and are useful as receptor antagonists (Gong JH et al., 1996; WO 99/16877). Alternatively, N-terminal extension of the mature CC-chemokine with one Methionine results in almost complete inactivation of the molecule, which also behaves as an antagonist for the authentic one (WO 96/17935).

Moreover, in order to perform structure-function analysis of CC-chemokines, variants containing substitutions or chemical modifications in different internal positions, as well as CC-chemokine derived peptides, have been tested for the interactions with receptors or other molecules. Some of these variants have been disclosed as having significatively altered binding properties, and sometimes they are active as CC-chemokine antagonists, having potential therapeutic applications in the treatment of HIV infection and some inflammatory or allergic diseases (WO 99/33989; Nardese V et al., 2001). In particular, the binding determinants and the physiological

relevance of the interactions of chemokines, by the means of specifically positioned basic residues, with Glycosaminoglycans (GAGs) has been intensively studied (WO 02/28419; Vives R et al., 2002; McCornack MA et al., 2002; Stringer SE et al., 2002; Stura E et al., 2002; Fukui S et al., 2002; Laurence JS et al., 2001; Martin L et al., 2001; Koopmann W and Krangel MS, 1997).

Even though there are potential drawbacks in using chemokines as therapeutic agents (tendency to aggregate, promiscuous binding), these molecules offer the possibility for therapeutic intervention in pathological conditions associated to such processes, in particular by inhibiting / antagonizing specific chemokines and their receptors at the scope to preventing the excessive recruitment and activation of cells, in particular leukocytes, for a variety of indications related to inflammatory and autoimmune diseases, cancers, and bacterial or viral infections (Schneider GP et al., 2001, Baggiolini M, 2001; Godessart N and Kunkel SL, 2001).

The possible therapeutic applications of chemokine-related compounds against hepatic diseases have been intensively studied, as recently reviewed (Ajuebor MN et al., 2002; Marra F, 2002; Colletti LM, 1999). In particular, it has been demonstrated that some chemokines are highly expressed and important for the recruitment for liver-infiltrating lymphocytes in hepatitis-related animal models (acetaminophen-induced, Concanavalin A-induced, adenovirus-induced, or hepatitis B virus-specific), suggesting a specific role of these molecules in the development of hepatitis (Katsumitsu A et al., 2002; Bautista AP, 2002; Dambach D et al., 2002; Lalor PF et al., 2002; Kakimi K et al, 2001; Tamaru M et al., 2000; Hogaboam CM et al., 2000; Kusano F et al., 2000).

Some broad spectrum CC-chemokine antagonists were disclosed in connection to hepatic diseases (WO 00/73327; W0 01/58916; US6495515). However, prior art fails to describe any therapeutic efficacy of an isolated, specific CC-chemokine mutant

generated by the substitution of internal residues, against liver inflammatory and/or fibrotic diseases.

SUMMARY OF THE INVENTION

It has been surprisingly found that a CCL5/RANTES mutant having reduced GAG-binding properties, resulting from the substitution of specific internal residues, counteracts liver injury in a relevant animal model. These evidences demonstrate the possibility of using this and other mutants having similar reduced GAG-binding activity in the treatment of liver inflammatory and/or fibrotic diseases. Particularly preferred are CCL5 mutants are the GAG-binding defective mutants of CCL5 generated by appropriately mutagenising the GAG-binding domain of CCL5.

Other features and advantages of the invention will be apparent from the following detailed description.

DESCRIPTION OF THE FIGURES

Figure 1: effect of the treatment with a GAG-binding defective CCL5 mutant (triple 40's RANTES mutant) in an animal model for liver diseases.

DETAILED DESCRIPTION OF THE INVENTION

The main object of the present invention is the use of a CC-chemokine mutant having a reduced GAG-binding activity for the treatment of liver inflammatory and/or fibrotic diseases. In particular, such mutants are the ones already disclosed in the prior art for the CC-chemokines CCL2/MCP-1, CCL3/MIP-1alpha, CCL4/MIP-1beta, or CCL5/RANTES (WO 02/28419; US. Appl. No. 60/371,442; Laurence JS et al., 2001; Koopmann W and Krangel MS, 1997).

In particular, the CC-chemokine mutants have the sequence of the ones disclosed in the prior art under the names of triple 40's RANTES (SEQ ID NO: 1) and MCP-1WT*2A (SEQ ID NO: 2) mutants. Similar experimental evidences have also been generated in connection with mutants of MIP-1alpha and MIP-1beta (Koopmann W and Krangel MS, 1997; Laurence JS et al., 2001). It is however evident that any other corresponding CC-chemokine mutant having reduced GAG-binding properties resulting from the substitution of the same residues disclosed in the prior art but with a different amino acid (i.e. the basic residue is substituted with a non-polar amino acid other than Ala or the acid residue), or resulting from a substitution in other position(s) are intended within the scope of the invention.

The above cited prior art on GAG-binding defective CC-chemokine mutants fails to identify liver inflammatory and/or fibrotic diseases as therapeutic indications in which these molecules can provide a beneficial effect. As there are currently therapies only partially effective and/or acceptable for treating diseases such as alcoholic liver diseases, viral or autoimmune hepatitis, the disclosed CC-chemokine mutants represent alternative therapeutic compounds possibly better accepted and efficient than the current therapies

The wording "a reduced GAG-binding activity" or "GAG-binding defective" means that the CC-chemokine mutants have a lower ability to bind to GAGs, i. e. a lower percentage of each of these mutants bind to GAGs (like heparin sulphate) with respect to the corresponding wild-type molecule, as measured with the assays in the above cited prior art disclosing such mutants.

In addition to the mutation at the specific positions leading to the decreased affinity for GAGs, the CC-chemokine mutants may include other modifications with respect to the wild-type molecule, generating active mutants of said CC-chemokine

mutants in which one or more amino acids have been added, deleted, or substituted. These additional modifications should be intended to maintain, or even improve, the properties of the mutants characterized in the present invention, or by any other relevant means known in the art, making them equally useful for treating liver inflammatory and/or fibrotic diseases.

These polypeptides can be prepared by chemical synthesis, by site-directed mutagenesis techniques, or any other known technique suitable thereof, which provide a finite set of substantially corresponding mutated or shortened peptides or polypeptides which can be routinely obtained and tested by one of ordinary skill in the art using the teachings presented in the prior art and in the Examples of the present patent application. Similar compounds may also result from conventional mutagenesis technique of the encoding DNA, from combinatorial technologies at the level of encoding DNA sequence (such as DNA shuffling, phage display/selection), or from computer-aided design studies based on the tridimensional structure and other functional assays of chemokines, with or without the presence of GAGs (Rajarathnam K, 2002; Vives R et al., 2002; McCornack MA et al., 2002; Stringer SE et al., 2002; Stura E et al., 2002; Fukui S et al., 2002 ; Martin L et al., 2001).

In accordance with the present invention, other additional preferred changes in these active mutants are commonly known as "conservative" or "safe" substitutions, that is, with amino acids having sufficiently similar chemical properties, in order to maintain the structure and the biological function of the CC-chemokine mutant. It is clear that insertions and deletions of amino acids may also be made in the above defined sequences without altering their function, particularly if the insertions or deletions only involve a few amino acids, e.g., under ten, and preferably under three,

and do not remove or displace amino acids which are critical to the functional conformation of a protein or a peptide.

The literature provide many models on which the selection of conservative amino acids substitutions can be performed on the basis of statistical and physico-chemical studies on the sequence and/or the structure of natural protein (Rogov SI and Nekrasov AN, 2001). Protein design experiments have shown that the use of specific subsets of amino acids can produce foldable and active proteins, helping in the classification of amino acid "synonymous" substitutions which can be more easily accommodated in protein structure, and which can be used to detect functional and structural homologs and paralogs (Murphy LR et al., 2000). The synonymous amino acid groups and more preferred synonymous groups are those defined in Table I.

Alternatively, active CC-chemokine mutants may contain on or more non-natural, amino acid derivatives being "synonymous" to a natural amino acid, are those defined in Table II. By "amino acid derivative" is intended an amino acid or amino acid-like chemical entity other than one of the 20 genetically encoded naturally occurring amino acids. In particular, the amino acid derivative may contain substituted or non-substituted alkyl linear, branched, or cyclic moieties, and may include one or more heteroatoms. The amino acid derivatives can be made de novo or obtained from commercial sources (Calbiochem-Novabiochem AG, Switzerland; Bachem, USA). Various methodologies for incorporating unnatural amino acids derivatives into proteins, using both *in vitro* and *in vivo* translation systems, to probe and/or improve protein structure and function are disclosed in the literature (Dougherty DA, 2000).

The term "active" means that such alternative compounds should maintain the therapeutic properties of the CC-chemokines mutants against liver inflammatory and/or

fibrotic diseases as described in the present invention, and should be as well pharmaceutically acceptable and useful.

In another embodiment, a polypeptide comprising the GAG-binding defective CC-chemokine mutant and an amino acid sequence belonging to a protein sequence other than the corresponding CC-chemokine can be also used for treating liver inflammatory and/or fibrotic diseases. The heterologous sequence is intended provide additional properties without considerably impairing the therapeutic activity. Examples of such additional properties are an easier purification procedure, a longer lasting half-life in body fluids, an additional binding moiety, the maturation by means of an endoproteolytic digestion, or extracellular localization. This latter feature is of particular importance for defining a specific group of fusion or chimeric proteins included in the above definition since it allows the CC-chemokine mutants to be localized in the space where not only where the isolation and purification of these polypeptides is facilitated, but also where CC-chemokines naturally interact with receptors and other molecules. Design of the moieties, ligands, and linkers, as well methods and strategies for the construction, purification, detection and use of fusion proteins are widely discussed in the literature (Nilsson J et al., 1997; "Applications of chimeric genes and hybrid proteins" Methods Enzymol. Vol. 326-328, Academic Press, 2000; WO 01/77137).

Additional protein sequences which can be used to generate the antagonists of the present invention can be chosen amongst extracellular domains of membrane-bound protein, immunoglobulin constant regions, multimerization domains, extracellular proteins, signal peptide-containing proteins, export signal-containing proteins. The choice of one or more of these sequences to be fused to the CC-chemokine mutants of the invention is functional to the desired use, delivery and/or preparation method.

The GAG-binding defective CC-chemokine mutants can be also provided for the treatment of liver inflammatory and/or fibrotic diseases in the form of the corresponding active precursors, salts, derivatives, conjugates or complexes. These alternative forms may be preferred according to the desired method of delivery and/or production.

The "precursors" are compounds which can be converted into the compounds of present invention by metabolic and enzymatic processing prior or after the administration to the cells or to the organism.

The term "salts" herein refers to both salts of carboxyl groups and to acid addition salts of amino groups of the peptides, polypeptides, or analogs thereof, of the present invention. Salts of a carboxyl group may be formed by means known in the art and include inorganic salts, for example, sodium, calcium, ammonium, ferric or zinc salts, and the like, and salts with organic bases as those formed, for example, with amines, such as triethanolamine, arginine or lysine, piperidine, procaine and the like. Acid addition salts include, for example, salts with mineral acids such as, for example, hydrochloric acid or sulfuric acid, and salts with organic acids such as, for example, acetic acid or oxalic acid. Any of such salts should have substantially similar activity to the peptides and polypeptides of the invention or their analogs.

The term "fractions" as herein used refers to derivatives which can be prepared from the functional groups present on the lateral chains of the amino acid moieties or on the N-/ or C-terminal groups according to known methods. Such derivatives include for example esters or aliphatic amides of the carboxyl-groups and N-acyl derivatives of free amino groups or O-acyl derivatives of free hydroxyl-groups and are formed with acyl-groups as for example alcanoyl- or aroyl-groups. Alternatively, the derivatives may contain sugars or phosphates groups linked to the functional groups present on the lateral chains of the amino acid moieties. Such molecules can result from *In vivo* or *in*

vitro processes which do not normally alter primary sequence, for example chemical derivativization of peptides (acetylation or carboxylation), phosphorylation (introduction of phosphotyrosine, phosphoserine, or phosphothreonine residues) or glyco sylation (by exposing the peptide to enzymes which affect glycosylation e.g., mammalian glycosylating or deglycosylating enzymes).

The term "derivatives" as herein used refers to derivatives which can be prepared from the functional groups present on the lateral chains of the amino acid moieties or on the N- or C-terminal groups according to known methods. Such derivatives include for example esters or aliphatic amides of the carboxyl-groups and N-acyl derivatives of free amino groups or O-acyl derivatives of free hydroxyl-groups and are formed with acyl-groups as for example alcanoyl- or aroyl-groups. Alternatively, useful conjugates or complexes of the CC-chemokine mutants can be generated by using molecules and methods known in the art for improving the detection of the interaction with other proteins (radioactive or fluorescent labels, biotin), therapeutic efficacy (cytotoxic agents, isotopes), or drug delivery efficacy, such as polyethylene glycol and other natural or synthetic polymers (Pillai O and P anchagnula R, 2001). In the latter case, a site-directed modification of an appropriate residue, present in the natural sequence or introduced by mutating the natural sequence, at an internal or terminal position, can be introduced. Similar modifications have been already disclosed for chemokines (WO 02/04499; WO 02/04015; Vita C et al., 2002).

Any residue can be used for attachment, provided it has a side-chain amenable for polymer attachment (i.e., the side chain of an amino acid bearing a functional group, e.g., lysine, aspartic acid, glutamic acid, cysteine, histidine, etc.). Alternatively, a residue at these sites can be replaced with a different amino acid having a side chain amenable for polymer attachment. Polymers suitable for these purposes are

biocompatible, namely, they are non-toxic to biological systems, and many such polymers are known. Such polymers may be hydrophobic or hydrophilic in nature, biodegradable, non-biodegradable, or a combination thereof. These polymers include natural polymers (such as collagen, gelatin, cellulose, hyaluronic acid), as well as synthetic polymers (such as polyesters, polyorthoesters, polyanhydrides). Examples of hydrophobic non-degradable polymers include polydimethyl siloxanes, polyurethanes, polytetrafluoroethylenes, polyethylenes, polyvinyl chlorides, and polymethyl methaerylates. Examples of hydrophilic non-degradable polymers include poly(2-hydroxyethyl methacrylate), polyvinyl alcohol, poly(N-vinyl pyrrolidone), polyalkylenes, polyacrylamide, and copolymers thereof. Preferred polymers comprise as a sequential repeat unit ethylene oxide, such as polyethylene glycol (PEG).

The preferred method of attachment employs a combination of peptide synthesis and chemical ligation. Advantageously, the attachment of a water-soluble polymer will be through a biodegradable linker, especially at the N-terminal region of a protein. Such modification acts to provide the protein in a "pro-drug" form, that, upon degradation of the linker releases the protein without polymer modification.

The GAG-binding defective CC-chemokine mutants may be prepared by any appropriate procedure in the art, such as recombinant DNA-related technologies involving the expression in Eukaryotic cells (e.g. yeasts, insect or mammalian cells) or Prokaryotic cells. Detailed methods for producing the GAG-binding defective CC-chemokine mutants can be found in the prior art originally disclosing them (WO 02/28419; US. Appl. No. 60/371,442), as well as in other literature featuring protocols for chemokine production (Edgerton MD et al., 2000) or common molecular biology techniques for the production of recombinant proteins in Prokaryotic or Eukaryotic host cells, such as some titles in the series "A Practical Approach" published by Oxford

University Press ("DNA Cloning 2: Expression Systems", 1995; "DNA Cloning 4: Mammalian Systems", 1996; "Protein Expression", 1999; "Protein Purification Techniques", 2001).

Alternatively the GAG-binding defective CC-chemokine mutants may be prepared by any other well known procedure in the art, in particular, by the well established chemical synthesis procedures, which can be efficiently applied on these molecule given the short length. Totally synthetic chemokines, also containing additional chemical groups, are disclosed in the literature (Brown A et al., 1996; Vita C et al., 2002).

Examples of chemical synthesis technologies are solid phase synthesis and liquid phase synthesis. As a solid phase synthesis, for example, the amino acid corresponding to the N-terminus of the peptide to be synthetized is bound to a support which is insoluble in organic solvents, and by alternate repetition of reactions, one wherein amino acids with their amino groups and side chain functional groups protected with appropriate protective groups are condensed one by one in order from the C-terminus to the N-terminus, and one where the amino acids bound to the resin or the protective group of the amino groups of the peptides are released, the peptide chain is thus extended in this manner. Solid phase synthesis methods are largely classified by the tBoc method and the Fmoc method, depending on the type of protective group used. Typically used protective groups include tBoc (t-butoxycarbonyl), Cl-Z (2-chlorobenzyloxycarbonyl), Br-Z (2-bromobenzyloxycarbonyl), Bzl (benzyl), Fmoc (9-fluorenylmethoxycarbonyl), Mbh (4,4'-dimethoxydibenzhydryl), Mtr (4-methoxy-2,3,6-trimethylbenzenesulphonyl), Trt (trityl), Tos (tosyl), Z (benzyloxycarbonyl) and Cl2-Bzl (2,6-dichlorobenzyl) for the amino groups; NO2 (nitro) and Pmc (2,2,5,7,8-pentamethylchromane-6-sulphonyl) for the guanidino

groups); and tBu (t-butyl) for the hydroxyl groups). After the synthesis, the desired peptide is subjected to the de-protection reaction and cut out from the solid support. Such peptide cutting reaction may be carried with hydrogen fluoride or tri-fluoromethane sulfonic acid for the Boc method, and with TFA for the Fmoc method. Finally, the intact full-length peptides are purified and chemically or enzymatically folded (including the formation of disulphide bridges between cysteines) into the corresponding CC-chemokine mutants.

Purification of the natural, synthetic or recombinant proteins is carried out by any one of the methods known for this purpose, i.e. any conventional procedure involving extraction, precipitation, chromatography, electrophoresis, or the like. A further purification procedure that may be used in preference for purifying the protein of the invention is affinity chromatography using monoclonal antibodies, heparin, or any other suitable ligand which can bind the target protein at high efficiency and can be immobilized on a gel matrix contained within a column. Impure preparations containing the proteins are passed through the column. The protein will be bound to the column by means of this ligand while the impurities will pass through. After washing, the protein is eluted from the gel by a change in pH or ionic strength. Alternatively, HPLC (High Performance Liquid Chromatography) can be also used.

Another object of the present invention is the use of a CC-chemokine mutant having reduced GAG-binding activity in the preparation of a pharmaceutical composition for liver inflammatory and/or fibrotic diseases, in particular when formulated in combination with pharmaceutically acceptable carriers, excipients, stabilizers, adjuvants, or diluents.

A non-limitative list of disorders involving hepatic damage in which the CC-chemokine mutant having reduced GAG-binding activity can be used includes alcoholic

liver diseases (cirrohosis, steatosis), a viral hepatitis, an autoimmune hepatitis, or any other liver fibrotic degeneration.

Still another object of the present invention are methods for the treatment or prevention of a liver inflammatory and/or fibrotic disease, comprising the administration of an effective amount of a CC-chemokine mutant having reduced GAG-binding activity.

The CC-chemokine mutants may be used alone, or with another therapeutic composition acting synergically or in a coordinated manner with them in the treatment of liver inflammatory and/or fibrotic diseases. For example, similar synergistic properties of CC-chemokine mutants have been demonstrated in combination with cyclosporin (WO 00/16796).

In view of the claimed uses, any drug delivery method allowing the targeting of the GAG-binding defective CC-chemokine mutant is preferred. Similar methods are known in the prior and may involve the conjugation of the CC-chemokine mutant with galactosylated or mannosylated albumin (Chuang VT et al., 2002) or the synthesis of polymeric nanoparticles from a sugar-containing conjugate composed of lactobionic acid, diamine-terminated polyethylene glycol) and cholic acid (Kim IS and Kim SH, 2002).

An "effective amount" refers to an amount of the active ingredients that is sufficient to affect the course and the severity of the disease, leading to the reduction or remission of the liver pathology. The effective amount will depend on the route of administration and the condition of the patient.

The pharmaceutical compositions may be formulated in any acceptable way to meet the needs of the mode of administration for treating liver diseases. For example, the use of biomaterials and other polymers for drug delivery, as well the different

techniques and models to validate a specific mode of administration, are disclosed in literature (Luo B and Prestwich GD, 2001; Cleland JL et al., 2001).

"Pharmaceutically acceptable" is meant to encompass any carrier, which does not interfere with the effectiveness of the biological activity of the active ingredient and that is not toxic to the host to which is administered. Carriers can be selected also from starch, cellulose, talc, glucose, lactose, sucrose, gelatin, malt, rice, flour, chalk, silica gel, magnesium stearate, sodium stearate, glycerol monostearate, sodium chloride, dried skim milk, glycerol, propylene glycol, water, ethanol, and the various oils, including those of petroleum, animal, vegetable or synthetic origin (peanut oil, soybean oil, mineral oil, sesame oil). For example, for parenteral administration, the above active ingredients may be formulated in unit dosage form for injection in vehicles such as saline, dextrose solution, serum albumin and Ringer's solution.

Besides the pharmaceutically acceptable carrier, the compositions of the invention can also comprise minor amounts of additives, such as stabilizers, excipients, buffers and preservatives which may facilitate the processing of the active compounds into preparations which can be used pharmaceutically. Moreover, these compositions may contain another active ingredient which can act synergically or in a coordinated manner with the CC-chemokine mutants of the invention.

The administration of such active ingredient may be by intravenous, intramuscular or subcutaneous route. Other routes of administration, which may establish the desired effects of the respective ingredients in the liver, are comprised by the present invention. For example, administration may be by various parenteral routes such as subcutaneous, intravenous, intradermal, intramuscular, intraperitoneal, intranasal, transdermal, oral, or buccal routes. The pharmaceutical compositions of the present invention can also be administered in sustained or controlled release dosage

forms, including depot injections, osmotic pumps, and the like, for the prolonged administration of the polypeptide at a predetermined rate, preferably in unit dosage forms suitable for single administration of precise dosages.

Parenteral administration can be by bolus injection or by gradual perfusion over time. Preparations for parenteral administration include sterile aqueous or non-aqueous solutions, suspensions, and emulsions, which may contain auxiliary agents or excipients known in the art, and can be prepared according to routine methods. In addition, suspension of the active compounds as appropriate oily injection suspensions may be administered. Suitable lipophilic solvents or vehicles include fatty oils, for example, sesame oil, or synthetic fatty acid esters, for example, sesame oil, or synthetic fatty acid esters, for example, ethyl oleate or triglycerides. Aqueous injection suspensions that may contain substances increasing the viscosity of the suspension include, for example, sodium carboxymethyl cellulose, sorbitol, and/or dextran. Optionally, the suspension may also contain stabilizers. Pharmaceutical compositions include suitable solutions for administration by injection, and contain from about 0.01 to 99.99 percent, preferably from about 20 to 75 percent of active compound together with the excipient.

The optimal dose of active ingredient may be appropriately selected according to the route of administration, patient conditions and characteristics (sex, age, body weight, health, size), extent of symptoms, concurrent treatments, frequency of treatment and the effect desired. Adjustment and manipulation of established dosage ranges are well within the ability of those skilled.

Usually a daily dosage of active ingredient can be about 0.01 to 100 milligrams per kilogram of body weight. Ordinarily 1 to 40 milligrams per kilogram per day given in divided doses or in sustained release form is effective to obtain the desired results.

Second or subsequent administrations can be performed at a dosage, which is the same, less than, or greater than the initial or previous dose administered to the individual.

The present invention has been described with reference to the specific embodiments, but the content of the description comprises all mo difications and substitutions, which can be brought by a person skilled in the art without extending beyond the meaning and purpose of the claims.

The Invention will now be described by means of the following Examples, which should not be construed as in any way limiting the present in vention. The Examples will refer to the Figures specified here below.

EXAMPLES

Example 1: efficacy of different CCL5 mutants in a hepatitis model

Concanavalin A (Con A)-induced liver injury depends on the activation of recruitment of CD4(+) T cells by macrophages. In general, T cells are the driving force underlying immunologically mediated hepatic disorders, making therefore the Con -A model highly relevant for studying the pathophysiology of diseases such as autoimmune or acute hepatitis (Takeda K et al., 2000; Tiegs G et al., 1992).

The assay involves the measurement of serum alanine aminotransferase (ALT), a liver enzyme contained in hepatocytes and released into serum when these cells are damaged. ALT is the most widely used marker in humans and animal s to document damage and destruction of liver cells (as in hepatitis), and, generally, ALT concentration correlates with histological changes.

As example of CCL5 mutants acting as CCL5 antagonist, the triple 40's RANTES mutant and Met-RANTES were chosen to be expressed in *E. coli* and purified as previously described (WO 02/28419; WO 96/17935).

Specific pathogen-free male C57BL/6 mice (body weight of 21-23 grams; Charles River Breeding Farms) were treated with Phosphate Buffer Saline (PBS; vehicle control; 0.1 ml), or with one of above described CCL5 mutants (30 micrograms/mouse in 0.1 ml PBS). The mice (5-10 per group) were injected s.c. 1 hour prior to Con-A i.v. injection (freshly prepared Con A type V, 0.25 mg/mouse in 0.1 ml PBS; Sigma). At 8 hours after Con-A administration and under halothane anaesthesia, blood was collected for measuring plasma ALT using a commercial kit for ALT determination (Sigma).

This biochemical approach for quantifying liver injury showed that only the substituted CCL5 triple mutant, and not the N-terminal modified CCL5 mutant, is capable of reducing ALT concentration in serum of the pre-treated animals in a significant manner (figure 1).

The analysis of the data generated with the substituted CCL5 mutant, and not another CCL5-based antagonistic mutant, demonstrate how, using a single CC-chemokine mutant having the specific binding profile characterized in the prior art (WO 02/28419; US. Appl. No. 60/371,442), a surprising therapeutically relevant effect can be obtained, suggesting the use of similar molecules in the treatment of liver diseases involving inflammation or fibrosis. The comparison with results obtained using other animal models for such diseases (acetaminophen-induced or adenovirus-induced hepatitis), may provide further confirmation of the therapeutic applicability of these specific CC-chemokine mutants.

TABLE I

Amino Acid	Synonymous Group	More Preferred Synonymous Groups
Ser	Gly, Ala, Ser, Thr, Pro	Thr, Ser
Arg	Asn, Lys, Gln, Arg, His	Arg, Lys, His
Leu	Phe, Ile, Val, Leu, Met	Ile, Val, Leu, Met
Pro	Gly, Ala, Ser, Thr, Pro	Pro
Thr	Gly, Ala, Ser, Thr, Pro	Thr, Ser
Ala	Gly, Thr, Pro, Ala, Ser	Gly, Ala
Val	Met, Phe, Ile, Leu, Val	Met, Ile, Val, Leu
Gly	Ala, Thr, Pro, Ser, Gly	Gly, Ala
Ile	Phe, Ile, Val, Leu, Met	Ile, Val, Leu, Met
Phe	Trp, Phe,Tyr	Tyr, Phe
Tyr	Trp, Phe,Tyr	Phe, Tyr
Cys	Ser, Thr, Cys	Cys
His	Asn, Lys, Gln, Arg, His	Arg, Lys, His
Gln	Glu, Asn, Asp, Gln	Asn, Gln
Asn	Glu, Asn, Asp, Gln	Asn, Gln
Lys	Asn, Lys, Gln, Arg, His	Arg, Lys, His
Asp	Glu, Asn, Asp, Gln	Asp, Glu
Glu	Glu, Asn, Asp, Gln	Asp, Glu
Met	Phe, Ile, Val, Leu, Met	Ile, Val, Leu, Met
Trp	Trp, Phe,Tyr	Trp

TABLE II

Amino Acid	Synonymous Group
Ser	D-Ser, Thr, D-Thr, allo-Thr, Met, D-Met, Met(O), D-Met(O), L-Cys, D-Cys
Arg	D-Arg, Lys, D-Lys, homo-Arg, D-homo-Arg, Met, Ile, D-.Met, D-Ile, Orn, D-Orn
Leu	D-Leu, Val, D-Val, AdaA, AdaG, Leu, D-Leu, Met, D-Met
Pro	D-Pro, L-I-thioazolidine-4-carboxylic acid, D-or L-1-oxazolidine-4-carboxylic acid
Thr	D-Thr, Ser, D-Ser, allo-Thr, Met,D-Met, Met(O), D-Met(O), Val, D-Val
Ala	D-Ala, Gly, Aib, B-Ala, Acp, L-Cys, D-Cys
Val	D-Val, Leu, D-Leu, Ile, D-Ile, Met, D-Met, AdaA, AdaG
Gly	Ala, D-Ala, Pro, D-Pro, Aib, .beta.-Ala, Acp
Ile	D-Ile, Val, D-Val, AdaA, AdaG, Leu, D-Leu, Met, D-Met
Phe	D-Phe, Tyr, D-Thr, L-Dopa, His, D-His, Trp, D-Trp, Trans-3,4, or 5-phenylproline, AdaA, AdaG, cis-3,4, or 5-phenylproline, Bpa, D-Bpa
Tyr	D-Tyr, Phe, D-Phe, L-Dopa, His, D-His
Cys	D-Cys, S--Me--Cys, Met, D-Met, Thr, D-Thr
Gln	D-Gln, Asn, D-Asn, Glu, D-Glu, Asp, D-Asp
Asn	D-Asn, Asp, D-Asp, Glu, D-Glu, Gln, D-Gln
Lys	D-Lys, Arg, D-Arg, homo-Arg, D-homo-Arg, Met, D-Met, Ile, D-Ile, Orn, D-Orn
Asp	D-Asp, D-Asn, Asn, Glu, D-Glu, Gln, D-Gln
Glu	D-Glu, D-Asp, Asp, Asn, D-Asn, Gln, D-Gln
Met	D-Met, S--Me--Cys, Ile, D-Ile, Leu, D-Leu, Val, D-Val

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CLAIMS

1. Use of a CC-chemokine mutant having a reduced GAG-binding activity for the treatment of liver inflammatory and/or fibrotic diseases.

2. The use of claim 1 wherein the CC-chemokine is CCL2, CCL3, CCL4, or CCL5.

3. The use of claim 2 wherein the CC-chemokine mutant has the amino acid sequence of SEQ ID NO: 1.

4. The use of claim 2 wherein the CC-chemokine mutant has the amino acid sequence of SEQ ID NO: 2.

5. The use of any of the claims from 1 to 4 wherein the CC-chemokine mutant is an active mutant of said CC-chemokine mutant in which one or more amino acids have been added, deleted, or substituted.

6. The use of any of the claims from 1 to 5 wherein the CC-chemokine mutant is comprised in a polypeptide additionally comprising an amino acid sequence belonging to a protein sequence other than the corresponding CC-chemokine.

7. The use of any of the claims from 1 to 6 wherein the CC-chemokine mutant is in the form of an active precursors, salts, derivatives, conjugates or complexes.

8. Use of a CC-chemokine mutant having reduced GAG-binding activity in the preparation of a pharmaceutical composition for liver inflammatory and/or fibrotic diseases.

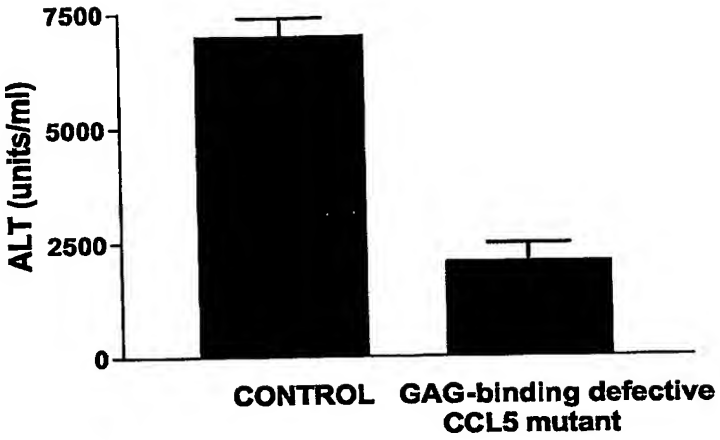
9. The use of any of the preceding claims wherein the liver disease is an alcoholic liver disease, a viral hepatitis, or an autoimmune hepatitis

10. Methods for the treatment or prevention of liver inflammatory and/or fibrotic diseases, comprising the administration of an effective amount of a CC-chemokine mutant having reduced GAG-binding activity.

ABSTRACT

CC-chemokine mutants having reduced Glycosaminoglycans (GAG)-binding properties are effective against liver inflammatory and/or fibrotic diseases. Particularly preferred are the mutants of CCL5/RANTES having reduced GAG-binding properties.

Figure 1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2500
0
ALT (units/ml)
CONTROL
GAG-binding defective CCL5 mutant

SEQUENCE LISTING

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Lys Glu Ile Cys Ala Asp Pro Lys Gln Lys Trp Val Gln Asp Ser Met
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Asp His Leu Asp Lys Gln Thr Gln Thr Pro Lys Thr
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